Amendment G4 to Applicant

Please amend the paragraph commencing at page 23, line 13, as follows.

G3

Figure 13 shows the sequence of the rabbit LIPG PCR product (RLLG.SEQ, SEQ ID NO: 12) and the sequence alignment between the rabbit LIPG PCR product and the corresponding sequence in the human cDNA (LLG7742A) (SEQ ID NO: 7). Identical nucleotides are shaded.

Please insert the following five paragraphs at page 25, between lines 7 and 8.

The present invention relates to a polypeptide encoded by the LIPG gene and any analogue, fragment, derivative, or mutant thereof which is derived from such polypeptide and which retains at least one biological property thereof (hereafter, collectively, "LIPG polypeptide", "LIPG protein", "endothelial lipase" or "EL", or "LLG"). The LIPG polypeptide is a member of the triacyglycerol lipase family which includes also hepatic lipase (HL), lipoprotein lipase (LPL), and pancreatic lipase (PL). Unique to LIPG polypeptide in comparison with the other members of the triacylglycerol lipase family is that it has been found to be synthesized by endothelial cells.

G4

The present invention relates also to compositions and methods for regulating the levels of HDL cholesterol and apolipoprotein AI, VLDL cholesterol and LDL cholesterol. The compositions and methods of the present invention function by raising or lowering the levels of LIPG polypeptide.

says activity - page 25, line 12

LIPG polypeptide has the ability to lower the levels of HDL cholesterol and apolipoprotein AI as well as the levels of VLDL cholesterol and LDL cholesterol. In addition, LIPG is abundantly expressed in the placenta and a role for this enzyme in development is possible, given the importance of lipid transport in fetal development

(Farese et al., *Trends Genet.*, 14: 115-120 (1998)). Given HDL's beneficial properties including the reduction of the risk of atherosclerotic cardiovascular disease, it is desirable to raise HDL levels in a patient by lowering the enzymatic activity of LIPG polypeptide. Given the correlation of high levels of LDL and VLDL and increased risk of atherosclerotic diseases, it is desirable to lower the level of these compounds in a patient afflicted with high levels thereof by raising the enzymatic activity of LIPG polypeptide.

The sequence of LIPG polypeptide includes several aforementioned features which are common to the members of the triacyglycerol lipase family: (A) a GXSXG motif; (B) a "catalytic triad"; (C) a heparin-binding region; and (D) a "lid region". As stated previously, the lid region forms an amphipathic helix covering the catalytic pocket of the enzyme (Winkler et al., *Nature*, 343: 771-774 (1990); van Tilbeurgh et al., *J. Biol. Chem.*, 269: 4626-4633 (1994)) and confers substrate specificity to the enzymes of the triacylglycerol lipase family (Dugi et al., *J. Biol. Chem.*, 270: 25396-25401 (1995)). The lid region diverges significantly between members of the triacylglycerol lipase family. The lid region of LIPG polypeptide consists of 19 residues and is three residues shorter and less amphipathic than those found in LPL and HL, which is consistent with the fact that LIPG polypeptide has a different enzymatic profile from LPL and HL. The sequence of the lid region of LIPG polypeptide is shown in Figure 6 (the sequence between two framing cysteine residues in the sequence indicated by a bold line). LIPG polypeptide comprises also an approximately 39 kD catalytic domain of the triacylglycerol lipase family, e.g., having the sequence SEQ ID NO: 10.

G4 Cont

There exist two major forms of LIPG polypeptide: LLGXL polypeptide and LLGN polypeptide, either of which may exist in glycosylated or non-glycosylated form. Human LLGXL polypeptide (which includes SEQ ID NO.: 8) has 500 amino

OK

acids and a molecular weight of approximately 55 kD. The human LLGXL polypeptide exhibits 43% similarity to human lipoprotein lipase and 37% similarity to human hepatic lipase. Human LLGN polypeptide (which includes SEQ ID NO.: 6) has 354 amino acids and a molecular weight of approximately 40 kD. It is believed that a 68 kD form of LIPG polypeptide is likely a glycosylated form of human LLGXL polypeptide. SEQ ID NO.: 6 contains the same first 345 amino terminal residues as does SEQ ID NO.: 8. This common sequence is SEQ ID NO.: 10. In addition to this common sequence, SEQ ID NO.: 6 contains an additional nine unique residues while SEQ ID NO.: 8 contains an additional 146 unique residues.--

Please delete the paragraphs commencing at page 25, line 9, and at page 26, line 6.

Please amend the paragraph commencing at page 96, line 10, as follows.

A commercially available lambda cDNA library derived from rabbit lung tissue (Clontech, Cat. #TL1010b) was used to isolate a fragment of the rabbit homolog of the LIPG gene. Five microliters of the stock library were added to 45 ml water and heated to 95°C for 10 minutes. The following were added in a final volume of 100 ml: 200 mM dNTPs, 20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 100 mM each primer DLIP774 and LLGgen2a, and 2.5 U Taq polymerase (GIBCO). The reaction was thermocycled 35 times with the parameters of: 15 seconds at 94°C, 20 seconds at 50°C and 30 seconds at 72°C. Ten microliters of the reaction were analyzed via agarose gel electrophoresis. A product of approximately 300 basepairs was detected. A portion (4 ml) of the reaction mix was used to clone the product via the TA cloning system. The insert of a resulting clone was sequenced (SEQ ID NO: 11). An alignment between the deduced rabbit amino acid sequence (SEQ ID NO: 12) and the corresponding sequence of the human cDNA is also shown in Figure 13. Of the

Support for G4 amendment as sited by Applicant

DETAILED DESCRIPTION OF THE INVENTION

The detailed description which follows sets forth the basis for the present invention, followed by a definitions section. Following the definitions section, the various compositions useful in the practice of the invention are discussed, followed by a discussion of the methods used to lower or raise the levels of LIPG activity.

Add G4

The Enzymatic Activity of the LIPG Gene Product

The present invention relates to methods for regulating the levels of HDL cholesterol and apolipoprotein AI, VLDL cholesterol and LDL cholesterol utilizing methods and compositions which lower or raise the activity of the LIPG lipase enzyme. In particular, the present invention is based in part on the discovery of the enzymatic activity of the polypeptide products of the LIPG gene on HDL cholesterol and apolipoprotein AI, VLDL cholesterol and LDL cholesterol. The polypeptide products of LIPG are members of the triacylglycerol lipase family and comprise an approximately 39 kD catalytic domain of the triacylglycerol lipase family, e,g., having the sequence SEQ ID NO: 10. Because this newly discovered lipase was found to be synthesized by endothelial cells and this is a unique feature compared with other members of the triacylglycerol lipase family, this lipase has been named "endothelial lipase" (EL). Because the LIPG gene will be discussed extensively in the sections which follow, EL will be hereinafter referred to as LIPG polypeptide, for the purposes of clarity. In general, the LIPG polypeptide is found in two major forms, referred to hereinafter as "the LLGN polypeptide" and "the LLGXL polypeptide." The LLGN

polypeptide, has 354 amino acids. The LLGXL polypeptide has 500 amino acids and exhibits 43% similarity to human lipoprotein lipase and 37% similarity to human hepatic lipase. As used herein, the term "LIPG polypeptide" or "LIPG protein" encompasses both LLGN and LLGXL.

The sequence of the LIPG polypeptide contains the characteristic GXSXG lipase motif, a conserved catalytic triad, a 19-residue lid region, conserved heparin and lipoprotein binding sites and 5 potential N-linked glycosylation sites. The region with the greatest sequence divergence in the triacylglycerol lipase family is the lid domain, which forms an amphipathic helix covering the catalytic pocket of the enzyme (Winkler et al., *Nature*, 343, 771-774 (1990); van Tilbeurgh et al., *J. Biol. Chem.*, 269, 4626-4633 (1994)) and confers substrate specificity to the enzymes of this family (Dugi et al., *J. Biol. Chem.*, 270, 25396-25401 (1995)). The 19-residue lid region of LIPG is three residues shorter and less amphipathic than those found in lipoprotein lipase and hepatic lipase, consistent with a different enzymatic profile. The predicted molecule weight of the mature protein is approximately 55 kD; a 68 kD form is likely to be a glycosylated form, whereas a 40 kD form may be the product of a specific proteolytic cleavage.

The LIPG polypeptide has the ability to lower the levels of HDL cholesterol and apolipoprotein AI as well as the levels of VLDL cholesterol and LDL cholesterol. It is well established that lowered HDL cholesterol levels result in increased susceptibility to atherosclerosis and increased levels of HDL cholesterol can dramatically reduce

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The LIPG polypeptide has the ability to lower the levels of HDL cholesterol and apolipoprotein AI as well as the levels of VLDL cholesterol and LDL cholesterol. It is well established that lowered HDL cholesterol levels result in increased susceptibility to atherosclerosis and increased levels of HDL cholesterol can dramatically reduce

susceptibility to atherosclerosis.

One physiologic role of LIPG may be to hydrolyse HDL phospholipid in peripheral tissues and in liver to facilitate selective uptake of HDL cholesteryl ester via the HDL receptor SR-BI (Kozarsky et al., *Nature*, 387, 414-417 (1997)). Another possible role is the facilitation of apoB-containing remnant lipoprotein uptake, similar to the role of hepatic lipase (Mahley et al., *J. Lipid Res.*, 40, 1-16 (1999)). In addition, LIPG is abundantly expressed in the placenta, and a role for this enzyme in development is possible, given the importance of lipid transport in fetal development (Farese et al., *Trends Genet.*, 14, 115-120 (1998)).

Based on HDL cholesterol's beneficial properties, it is desirable to raise HDL cholesterol levels by lowering the enzymatic activity of LIPG. Accordingly, the present invention is directed to methods and compositions which lower the activity of LIPG in the body by lowering the expression of the LIPG gene or lowering the enzymatic activity of the LIPG polypeptide.

Given the ability of the LIPG polypeptide to reduce the levels of VLDL cholesterol and LDL cholesterol and the studies demonstrating the correlation between high levels of these compounds and atherosclerotic diseases, it is desirable to lower the level of these compounds in a patient. Accordingly, the present invention additionally provides methods and compositions for increasing the expression of the LIPG gene and increasing the enzymatic activity of the LIPG polypeptides.

polypeptide or protein which is substantially free of those compounds that are normally associated therewith in its natural state (e.g., other proteins or polypeptides, nucleic acids, carbohydrates, lipids). "Isolated" is not meant to exclude artificial or synthetic mixtures with other compounds, or the presence of impurities which do not interfere with biological activity, and which may be present, for example, due to incomplete purification, addition of stabilizers, or compounding into a pharmaceutically acceptable preparation.

A molecule is **"antigenic"** when it is capable of specifically interacting with an antigen recognition molecule of the immune system, such as an immunoglobulin (antibody) or T cell antigen receptor. An antigenic polypeptide contains at least about 5, and preferably at least about 10, amino acids. An antigenic portion of a molecule can be that portion that is immunodominant for antibody or T cell receptor recognition, or it can be a portion used to generate an antibody to the molecule by conjugating the antigenic portion to a carrier molecule for immunization. A molecule that is antigenic need not be itself immunogenic, *i.e.*, capable of eliciting an immune response without a carrier.

"LLGN polypeptide" and **"LLGN protein"** mean a polypeptide including the sequence SEQ ID NO: 6, said polypeptide being glycosylated or non-glycosylated.

"LLGXL polypeptide" and **"LLGXL protein"** mean a polypeptide including the sequence SEQ ID NO: 8, said polypeptide being glycosylated or non-glycosylated.

"LIPG polypeptide" and **"LIPG protein"** describe the

lipase enzyme encoded by the LIPG gene and generically describes both the LLGN polypeptide and the LLGXL polypeptide.

"Endothelial lipase," or **"EL"**, refer to the lipase enzyme encoded by the LIPG gene and is equivalent to the term LIPG polypeptide.

The LIPG polypeptide or protein of the invention includes any analogue, fragment, derivative, or mutant which is derived from an LIPG polypeptide and which retains at least one biological property of the LIPG polypeptide. Different variants of the LIPG polypeptide exist in nature. These variants may be allelic variations characterized by differences in the nucleotide sequences of the structural gene coding for the protein, or may involve differential splicing or post-translational modification. The skilled artisan can produce variants having single or multiple amino acid substitutions, deletions, additions, or replacements. These variants may include, inter alia: (a) variants in which one or more amino acid residues are substituted with conservative or non-conservative amino acids, (b) variants in which one or more amino acids are added to the LIPG polypeptide, (c) variants in which one or more of the amino acids includes a substituent group, and (d) variants in which the LIPG polypeptide is fused with another polypeptide such as serum albumin. Other LIPG polypeptides of the invention include variants in which amino acid residues from one species are substituted for the corresponding residue in another species, either at conserved or non-conserved positions. In another embodiment, amino acid residues at

A **"neutralizing antibody"** is an antibody which can bind to an LIPG polypeptide and lower or eliminate the enzymatic activity of the LIPG polypeptide. These antibodies may be monoclonal antibodies or polyclonal antibodies. The present invention includes chimeric, single chain, and humanized antibodies, as well as Fab fragments and the products of an Fab expression library, and Fv fragments and the products of an Fv expression library.

An **"inhibitory molecule"** or **"inhibitor"** is a molecule which lowers or eliminates the expression of the LIPG polypeptide or which lowers or eliminates the enzymatic activity of the LIPG polypeptide.

An **"enhancer molecule"** or **"enhancer"** is a molecule which increases the expression of the LIPG polypeptide or which increases the enzymatic activity of the LIPG polypeptide.

A **"liposome"** is is an artificial or naturally-occurring phospholipid vesicle.

A **"cationic liposome"** is a liposome having a net positive electrical charge.

The sections which follow discuss the elements used in the claimed methods and compositions and the preferred embodiments of these elements.

Polypeptides

The present invention utilizes polypeptides encoded by LIPG which are members of the triacylglycerol lipase family, and which comprise a 39 kD catalytic domain of the triacylglycerol lipase family, e.g., having the sequence SEQ ID NO: 10. In certain embodiments of the present invention,

an isolated LIPG polypeptide comprising the sequence SEQ ID NO: 6 and having an apparent molecular weight of about 40 kD on a 10% SDS-PAGE gel is utilized. In another embodiment of the present invention, an isolated LIPG polypeptide comprising the sequence SEQ ID NO: 8 and having an apparent molecular weight of about 55 kD or 68 kD on a 10% SDS-PAGE gel is utilized. In yet another embodiment, the polypeptides utilized in the present invention are subfragments of these polypeptides. In still yet another embodiment, the polypeptides used in the present invention are antibodies capable of binding to an LIPG polypeptide.

The polypeptides and proteins utilized in the present invention may be recombinant polypeptides, natural polypeptides, or synthetic polypeptides, and may be of human, rabbit, or other animal origin. The polypeptides are characterized by a reproducible single molecular weight and/or multiple set of molecular weights, chromatographic response and elution profiles, amino acid composition and sequence, and biological activity.

The polypeptides utilized in the present invention may be isolated from natural sources, such as placental extracts, human plasma, or conditioned media from cultured cells such as macrophages or endothelial cells, by using the purification procedures known to one of skill in the art.

Alternatively, the polypeptides utilized in the present invention may be prepared utilizing recombinant DNA technology, which comprises combining a nucleic acid encoding the polypeptide thereof in a suitable vector, inserting the resulting vector into a suitable host cell, recovering the

cells were plated in the presence of phorbol 12-myristate 13-acetate (PMA, 40 ng/ml; Sigma) for 48 hours. The differentiated THP-1 cells were exposed for 24 hours to either oxLDL (50 μg/ml) or control medium. Total RNAs were collected and purified using standard procedures. Poly(A)$^+$ RNA was purified from total RNA using a poly-dT magnetic bead system (Promega). cDNA synthesis and PCR amplification were accomplished using protocols from the Differential Display kit, version 1.0 (Display Systems Biotechnology). The primer pairs that yielded the initial cDNA fragment of EL were downstream primer 7 (5'-TTTTTTTTTTGA-3') and upstream primer 15 (5'-GATCCAATCGC-3'). The amplification reaction was fractionated on a 6% nondenaturing acrylamide sequencing format gel and an amplification product found only in the reaction containing cDNA from THP-1 cells exposed to oxLDL was identified and excised from the gel. A reamplification using the same primers was performed and the product was excised and subcloned into the pCRII vector using the TA cloning system (Invitrogen). Insert sizes were determined using *Eco*RI digestions of the plasmids, and clones containing inserts of the approximate size of the original PCR product were sequenced using fluorescent dye-terminator reagents (Prism, Applied Biosystems) and an Applied Biosystems 373 DNA sequencer. We extended the cDNA sequence of the original, gel-excised cDNA using the 5'-RACE system (GIBCO). RNA (1 μg) from the THP-1 cells used initially in the differential display reactions was used in the 5'-RACE procedure using a gene-specific primer (5'-TAGGACATGCACAGTGTAATCTG-3') for first strand cDNA synthesis. We performed PCR amplification

of the cDNA using an anchor primer and gene-specific primer 2 (5'-GATTGTGCTGGCCACTTCTC-3'). This reaction (1 µl) was used in a nested re-amplification using the universal amplification primer (5'-CUACUACUACUAGGCCACGCGTCGACTAGTAC-3') and the gene-specific primer 3 (5'-GACACTCCAGGGACTGAAG-3') to increase levels of specific product for subsequent isolation. The reaction products were cloned into the pCRII vector from the TA cloning kit and determined the sequence. A human placental cDNA library (oligo dT and random primed) was obtained from Clontech and probed with the 5'-RACE reaction PCR product. The DNA from hybridizing clones was purified using LambdaSorb reagent (Promega). Inserts were excised from the phage DNA by digestion with *EcoRI*, subcloned into the *EcoRI* site of the Bluescript II SK plasmid vector (Stratagene) and sequenced.

Example 12 - Antibody Preparation

A 17-residue peptide (GPEGRLEDKLHKPKATC) was synthesized corresponding to residues 8-23 of the secreted LIPG gene product on a Model 433A peptide synthesizer (Applied Biosystems). Peptide (2 mg) was coupled to maleimide-activated keyhole limpet haemocyanin (2 mg) following the protocols included in the Imject Activated Immunogen Conjugation kit (Pierce Chemical). After desalting, one-half of the conjugate was emulsified with an equal volume of Freund's complete adjuvant (Pierce) and injected into a New Zealand White rabbit. Four weeks after the initial inoculation, a booster inoculation was administered with an emulsification made exactly as described above except for the

were transferred to nitrocellulose membranes and detected with rabbit anti-LIPG peptide antisera (1:5,000), with goat anti-rabbit peroxidase conjugated antisera (1:5,000; Boehringer) as the secondary antibody. The membranes were reacted with Renaissance chemiluminescent reagent (DuPont NEN) and exposed to Kodak XAR-2 film. A commercially prepared filter containing poly(A)$^+$ RNAs (3 μg each) from human heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas (Clontech)was hybridized with a radiolabelled fragment and processed as described above. Following autoradiography, the blot was stripped by washing in boiling 0.1xSSC, 0.1% SDS for 2x15 minutes at 65 °C and then probed as described above with a 1.4-kb cDNA fragment encoding human LPL. This fragment was obtained by RT-PCR of the THP-1 RNA (PMA and oxLDL treated) using the 5' LPL and 3' LPL primers 5'-ACCACCATGGAGAGCAAAGCCCTG-3' and 5'-CCAGTTTCAGCCTGACTTCTTATTC-3', respectively. After exposure to film, the membranes were stripped again and reprobed with a radiolabelled fragment of human β actin cDNA to normalize to RNA content.

Human umbilical vein endothelial cells (HUVEC) were negative for LPL mRNA expression as expected, but were found to constitutively express a high level of mRNA for the LIPG gene (Figure 9).

Human coronary artery endothelial cells (HCAEC) were also found to express the mRNA which was further upregulated on treatment of these cells with phorbol ester (Figure 9).